AD-A137 693 LIPOSOMAL-ENCAPSULATED STROMA-FREE HEMOGLOBIN AS A POTENTIAL BLOOD SUBSTITUTE(U) CALIFORNIA UNIV SAN FRANCISCO C A HUNT 27 MAR 81 DAMD17-79-C-9045 1/1

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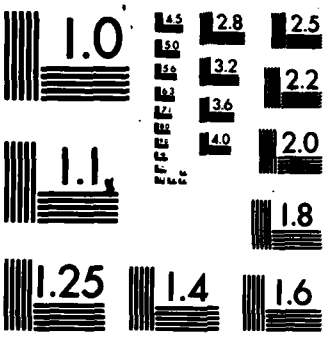

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Liposomal-Encapsulated Stroma-Free Hemoglobin as a Potential Blood Substitute

Annual Progress Report

by

C. Anthony Hunt, Ph.D.

March 27, 1981
(For the period May 1980 through April 1981)

Supported by

U.S. Army Medical Research and Development Command
Acquisition Group
Fort Detrick, Frederick, MD. 21701

Contract No. DAMD17-79-C-9045
University of California
San Francisco, California 94143

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A137 693 / 3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) LIPOSOMAL-ENCAPSULATED STROMA-FREE HEMOGLOBIN AS A POTENTIAL BLOOD SUBSTITUTE	5. TYPE OF REPORT & PERIOD COVERED Annual Progress Report May 1980 - April 1981
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) C. Anthony Hunt, Ph.D.	8. CONTRACT OR GRANT NUMBER(s) DAMD17-79-C-9045
9. PERFORMING ORGANIZATION NAME AND ADDRESS University of California San Francisco, California 94143	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 62772A.3S162772A874.AC.137
11. CONTROLLING OFFICE NAME AND ADDRESS US Army Medical Research and Development Command Fort Detrick Frederick, Maryland 21701	12. REPORT DATE March 27, 1981
	13. NUMBER OF PAGES 14
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)	15. SECURITY CLASS. (of this report) Unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

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SUMMARY

During the first year we described a new emulsion encapsulation procedure that dramatically increased efficiency of SFH encapsulation yielding HCL's ranging in size from 0.05 to 1.0μ in diameter. There was little or no binding of SFH to these HCL and O_2 binding properties were essentially the same as for solution of SFH. These HCL were less sensitive to osmotic shock than are RBC's and were stable for up to 72 hrs. at 4°C. Mild (3-6%) sucrose gradiants separated hemoglobin "rich" from hemoglobin poor HCL. In vivo studies indicated RES uptake was not the primary mechanism for blood clearance, rather extracellular binding and erosion are major processes which are saturable indicating that tissue uptake of transfusion should be a small fraction of the total.

During this second year we have overcome a major obstacle to further progress: scaling-up the emulsion encapsulation procedure to prepare large batches of HCL. The improved large scale procedure, which has advantages over the old micro-scale procedure, allows co-encapsulation of 2,4-dpg 9with 12% SFH, to-date) and procedures HCL with P50 values of 17-20. Further improvement may be possible. Results of a series of in vivo studies have been evaluated relative to the number, diameter and surface area of various liposome doses (same composition). Based on these studies a physiologically-based pharmacokinetic model has been developed which reasonably accounts for all data and indicates the feasibility of using HCL for transfusions. Other studies where in vivo stability, dose and size were controlled indicated that the circulation $t_{1/2}$ could be varied from 1.7 to 7.1 hrs. We are now able to prepare quantities of hemoglobin "rich" HCL suitable for transfusion studies in rats.

ANNUAL PROGRESS REPORT
TO THE
ACQUISITION GROUP
U.S. ARMY RESEARCH AND DEVELOPMENT COMMAND

LIPOSOME-ENCAPSULATED STROMA-FREE HEMOGLOBIN AS A POTENTIAL BLOOD SUBSTITUTE

C. Anthony Hunt

TYPE OF PROJECT

This proposal describes studies designed to evaluate hemoglobin-containing liposomes, HCL, as a component of a potential resuscitative fluid suitable for military field use. Additional studies are designed to optimize their properties as "artificial blood cells".

TECHNICAL OBJECTIVES

The technical objectives, as originally stated, are:

1. Quantify the O_2 binding properties of various new HCL, with and without diphosphoglycerate, relative to appropriate reference solutions of stroma-free hemoglobin at pH 7.4. Compare these results to data from similar studies where the inside pH of the HCL is reduced relative to the outside pH of 7.4 (inside pH ranging from 7.0 to 7.4.

2. Optimize the emulsion evaporation procedure, recently developed to produce HCL, as a function of lipid composition and hemoglobin concentration.

3. Determine the stability of HCL as a function of the ratio of lipid-to-trapped aqueous volume, as a function of the initial hemoglobin concentration, and as a function of time in buffer and plasma at various temperatures.

4. Prepare sufficient quantities of hemoglobin "rich" HCL so that USAMRDC can carry out animal (rat) transfusion studies.

5. Evaluate the disposition in mice of various HCL as a function of dose and HCL composition.

6. Evaluate the disposition of various HCL in an isolated perfused rat liver system using buffer and stroma-free hemoglobin solutions as perfusates, and document liver viability.

7. Conduct limited, repeat-dose toxicity studies of various HCL in mice.

8. Devise simplified HCL systems which may be useful under military field conditions.

We are now adding a new objective:

9. Using O_2 binding properties as well as *in vivo*/*in vitro* performance as quantifiable endpoints, optimize liposome composition relative to encapsulation of high percentages of stroma-free hemoglobin and 2,3-diphosphoglycerate, and to the relative proportion of hemoglobin "rich" vs. hemoglobin "poor" liposomes.

HYPOTHESIS

Our hypothesis is that stroma-free hemoglobin, SFH, encapsulated along with diphosphoglycerate in an appropriately designed liposome will be superior to simple solutions of SFH as a blood substitute. The function of SFH as a blood substitute may be dramatically improved if the rate of elimination of SFH were to be decreased and its retention in the circulation improved without adversely affecting oxygen binding properties. The available data, suggests that liposome encapsulation of SFH may provide these improvements.

Abbreviation Used:

HCL : hemoglobin-containing liposomes
SFH : stroma-free hemoglobin
Hb : hemoglobin
RES : reticuloendothelial system
FE : fluorocarbon emulsion
IPRLS: isolated perfused rat liver system
DPG: 2,3-diphosphoglycerate
PC : phosphatidylcholine
PA : phosphatidic acid
CH : cholesterol
aT : α-tocopherol
SG : sphingomyelin
PS : phosphatidylserine

BACKGROUND

In our original proposal we defined liposomes, summarized the literature relevant to our contention that hemoglobin-containing liposomes, HCL, suspended in a solution of SFH may be superior to SFH as a militarily useful blood substitute, and outlined our data on the development and evaluation of liposomes for in vivo use. At that time the idea seemed promising, but sufficient data was not available to make initial judgments. Our proposal was designed as a feasibility study to begin collecting such data.

The 1979-80 Progress Report contains substantial information. Discussions contained in that report should be referred to for background; to save space there is only limited carry-over to this report.

In our original proposal we identified a number of obstacles to the development of a useful HCL. These were:

1. The efficiency for encapsulating macromolecules, such as SFH, in liposomes has been generally poor relative to smaller solutes such as drugs. New techniques were needed to improve encapsulation.

2. The degree to which SFH may bind to and complex with liposomes and their lipids was unknown. Similarly, the O_2 binding properties of HCL were unknown.

3. The stability properties of HCL were unknown.

4. The available data suggested that liposomes in general may be rapidly cleared from the circulation by the RES, whereas a successful resuscitative fluid would require prolonged retention of liposomes in the circulation.

5. Because SFH had not been effectively encapsulated it was not clear whether sufficient oxygen binding capacity per unit of volume of solution could be delivered during a transfusion to make its use as a resuscitative fluid practical.

6. Liposome encapsulation of SFH may re-introduce "stromal-like properties" which give rise to toxicity.

Our progress and a mid-year report were presented at the Fall 1980 conference: "Current Concepts of Combat Casulaty Resuscitation". This report summarizes our progress to date. Over the past 10 months our work has focused in the following areas.

1. Further evaluation of dose-dependent nature of liposome disposition.

2. Evaluate the apparent $t_{1/2}$ of HCL in the circulation at relatively high, but sub-transfusion, doses.

3. Improve the emulsion evaporation procedure for preparation of HCL with and without DPG at various pH's.

4. Quantify HCL stability, and O_2 binding properties of the prepared HCL.

Why This Experimental Approach? After looking over the above adjectives one may ask: Why, at this stage, invest effort into evaluating dose-dependent liposome disposition, optimization of liposome preparation, etc.? Why not simply make a large batch of HCL, infuse them into a rat, and see if they work. We would if we could. Our reverse-phase HCL preparation procedure was worked out initially on a semi-micro scale (see the discussion below). Since additional information in the above areas will be needed in any case, we have selected to peruse the above goals in parallel with development of scaled-up techniques to prepare large batches of HCL.

1. Encapsulation Efficiency. We have applied a new procedure (4), emulsion evaporation, to the encapsulation of SFH. The procedure has no adverse effect on SFH when specific peroxide-free solvents are used and results in encapsulation of 20% of a 7% SFH solution when 200 M or 100 mg/ml of lipid (PC/PA/CH/aT in the molar ratio of 4/1/5/.1) is used - a ten-fold improvement over previously existing procedures (e.g. ref. 3). These HCL are not homogeneous with respect to aqueous volume per mg of lipid. Mild sucrose density gradients, which do not lyse the HCL, can separate hemoglobin "rich" (dark red) from hemoglobin "poor" (pink) liposomes. The lipid/aqueous volume ratio in the hemoglobin "rich" fraction is only 30% of that in the original HCL. Thus, the hemoglobin "rich" liposomes are composed of approximately 20% lipid by volume and 80% aqueous SFH. It is important to note that α-tocopherol is incorporated in all our HCL to minimize lipid oxidation both *in vivo* and *in vitro*; we have verified the importance of this.

Our efforts to improve HCL preparation procedures have so far generated more problems than they have solved. We have learned a lot, but, until recently, made little progress despite the investment of substantial effort.

Our problem stemmed from our early success. In our initial search for ways to increase the amount of trapped SFH solution per unit of lipid used, we selected the emulsion evaporation procedure. All of our early studies were carried out at the mg and ml level. This procedure has three major steps: (i) formation of an SFH solution in diethyl ether (selected because it gave no detectable SFH denaturation and because it could dissolve all the lipids) emulsion. (ii) removal of the organic phase to form a gel, and (iii) breaking of the gel to form HCL. Our problems came with attempts to scale-up this procedure to provide transfusion (animal) amounts of HCL. The micro-scale procedure required sonication to form the emulsion and produced about 0.5 ml of HCL. Good emulsion formation was (and is) essential. A long list of procedures to scale-up this procedure proved substantially less efficient than the micro-scale method; in each case the emulsion separated too rapidly and an unacceptable degree of SFH denaturation occurred.

We think we have now solved this problem. By using a (approximately 70/30) mixture of diethyl ether and trichlorotrifulorethane we have an organic phase with a density essentially identical to that of the aqueous SFH/DPG solution (other halocarbon solvents did not work). This system forms stable emulsions without sonication. Formation of methemoglobin, which forms aggregates with the lipids rather than being encapsulated, can be essentially eliminated by carrying out the emulsion formation under anoxic conditions. However, the procedure still yields a mixture of hemoglobin "rich' and hemoglobin "poor" liposomes.

2. Binding of SFH to Liposomes. Binding of SFH to the outside of liposomes is not significant for the liposome composition used. Binding of SFH within liposomes remains to be evaluated as does external binding when composition is changed. At this point binding does not appear to be a problem.

Oxygen binding properties of encapsulated SFH are not poorer than corresponding solutions of SFH. We should point out that one problem was encountered in these studies: the amount of Hb in aliquots of HCL as calculated from the amount of O_2 bound at 100% saturation was about twice the value obtained from direct spectrophotometric measures. The reason(s) for this discrepancy are being evaluated.

Typical O_2 binding results for our current HCL's are given in Table I.

TABLE I. Oxygen Binding Properties of HCL[a]

TYPE	[b]DPG/SFH	P50 at pH 7.4 (mm Hg)	n	Vol.%	g%SFH
6% SFH	No DPG	11.96	1.91	9.78	6.3
[c,d]6%SFH in HCL	2	18.01	1.93	2.03	[e]1.51
12% SFH	No DPG	13.35	2.48	18.05	12.47
[d]12% SFH in HCL	1	17.28	2.02	5.30	[f]4.0

[a]These two HCL preparations had the same composition mentioned above, only the concentration of encapsulated SFH and the amount of DPG was changed. [b]Molar ratio of DPG to SFH. [c]These HCL were stored at 4° for one week without detectable loss of SFH or DPG. [d]The hematocrit of these HCL was 1.0. [e]Based on sucrose encapsulation 75% of these HCL is aqueous but based on encapsulated SFH one would calculate that only 24% of the HCL volume is SFH at 6 g%; this is because these HCL are composed of hemoglobin "rich" and hemoglobin "poor" liposomes; SFH but not sucrose has been excluded from the latter. Thus, only in the hemoglobin rich liposomes does the encapsulated SFH concentration approach 6 or 12 g%. [f]See footnote 'e'. For these HCL 32%, rather than 24%, of the HCL volume is 12% SFH.

3. Stability of HCL. Our studies have shown that HCL of the above composition are not fragile. They are insensitive to mild osmotic shock and they retain their contents without significant leakage at 20° for 72 hours and 4° for at least one week. Thus, the stability of this composition HCL is not a major problem. Knowing that the stability of all liposomes is a direct function of their CH content, it will be of interest to assess stability at lower CH contents and, more important, in the presence of plasma. Our studies with liposomes of the same composition but without encapsulated SFH (in press, Int. J. Pharmac.) show that liposome interaction

with plasma components decreases as CH content increases. Further, after an initial binding of proteins to liposomes, which results in content leakage - again inversely proportional to CH content, a stabilized liposome is formed which loses its contents only very slowly.

4. RES Uptake of Liposomes. Clearance of liposomes by the RES is not the primary mechanism for the initial disposition of intact liposomes. Initial disposition is the result of binding to tissues, breakdown in the circulating fluids and slow RES uptake. More important, all of these processes are saturable.

It should be noted that suspensions of HCL and fluorocarbon emulsions, another potential blood substitute, have properties in common. Both are particulate suspensions and therefore both will be taken up to some extent by the RES. The dose used to evaluate the in vivo properties of the fluorocarbon emulsions has consistently been much larger than doses of liposomes similarly used. It is likely that the relatively long circulating life-time of the emulsion is the result of saturation of tissue binding and RES uptake.

Our HCL have consisted of one evolved composition: PC/PA/CH/aT in the molar ratio 4/1/5/0.1. Our preliminary data indicated that at high liposome doses (substantially lower than a transfusion dose) tissue binding became saturated and blood $t_{1/2}$ increased. Our understanding of these effects has now improved dramatically (see Figures 1-3). At constant composition these vesicles have organ distribution properties that are dependent on both dose and liposome size (we are the first laboratory to devise techniques to quantitate both the number and total surface area of a liposome dose). The data in Figures 1-3 show tissue levels 2 hrs. after various doses of three sizes of liposomes; quantitation is by co-encapsulated ^{14}C-inulin. The pattern seen is most coherent when percent dose in blood and liver is plotted vs. number of liposomes, and when percent dose in spleen is plotted vs. the surface area of the dose (in these figures the largest dose is an order of magnitude lower than a transfusion dose). Several significant observations can be made. (i) Saturation of tissue binding occurs at lower doses for for 0.3μ liposomes than for either larger or smaller (FP) liposomes. (ii) The retention of liposomes in the circulation depends on liver binding being saturated. (iii) Spleen saturation occurs, but at doses larger than is required to begin saturation of liver. (iv) There is a liposome diameter (> FP and <0.3) below which tissue disposition properties change. (v) HCL of 1.0μ diameter will have a shorter circulation $t_{1/2}$ than will HCL of 0.3μ diameter, yet the amount of SFH encapsulated per g of lipid is about the same for the two diameters.

Using our accumulated time-course data as a data base, we have developed a rational pharmacokinetic model to aid in describing and predicting the organ distribution properties of liposomes. This model is based on quantitation of an encapsulated aqueous (liposome) space maker such as inulin. C_1 is the percent of the dose remaining encapsulated and located outside liver and spleen; it includes blood and carcass levels. C_2 and C_3 are the percentage of the dose remaining encapsulated and located in blood in the liver or spleen. Extracellular free inulin is denoted as f_1; free inulin in the blood within liver or spleen is f_2 and f_3; free inulin elsewhere in blood is f_1. X_2 and X_3 are the percent dose remaining encapsulated, but bound extracellularly within liver and spleen; f_{x2} and f_{x3} represent any extracellularly bound free inulin. Y_2 and Y_3 represent the percent of dose irreversibly taken by cells in liver and spleen (inulin can not partition out of cells). The k_i are apparent first order rate constants; k_e is the known rate constant for excretion

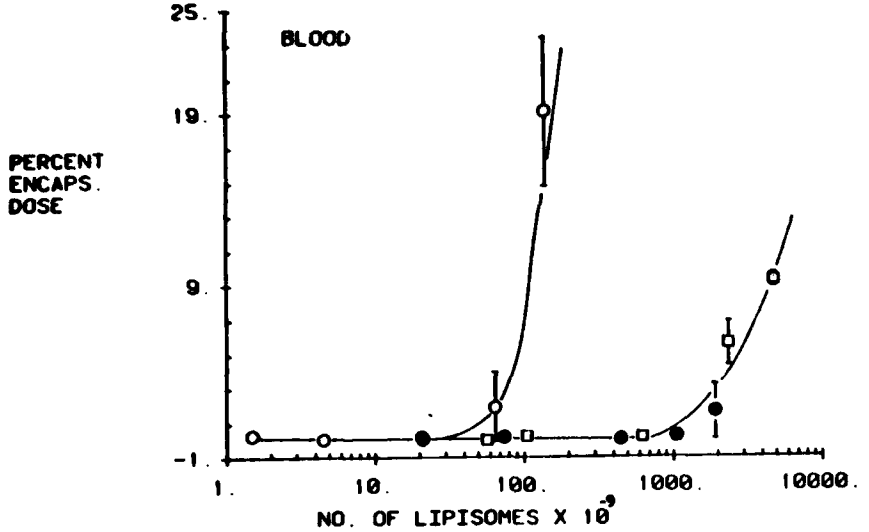


FIGURE 1. Percent of encapsulated ^{14}C inulin dose in blood (per ml). All liposomes were composed of PC/PA/CH/A-T (4/1/5/0.1). Doses of 1.0μ, 0.3μ and FP liposomes were used. All data are at two hrs. after the intravenous dose. The number of liposomes in each dose was determined. This data shows the most internal consistancy when plotted vs number of liposomes injected. Each value is mean of 3 animals (± S.D.). Liposome sizes: 1.0μ (●), 0.3μ (○) and FP (□).

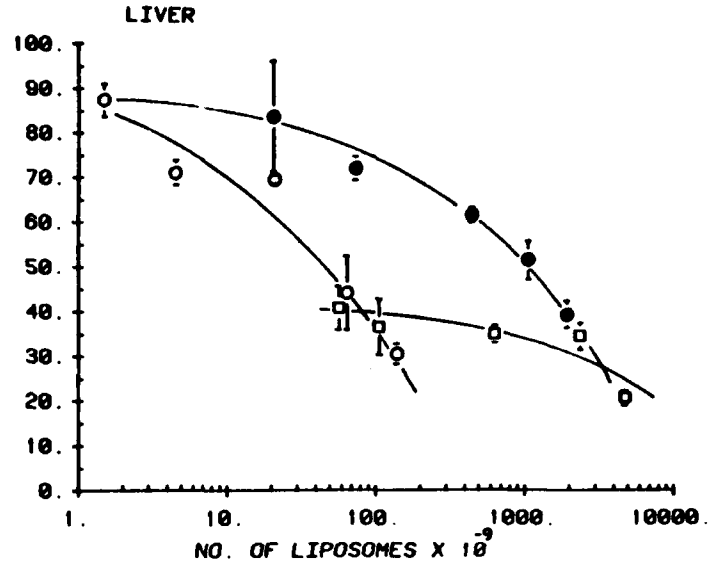


FIGURE 2. Percent of encapsulated ^{14}inulin dose in liver. See Fig. 1 for additional details.

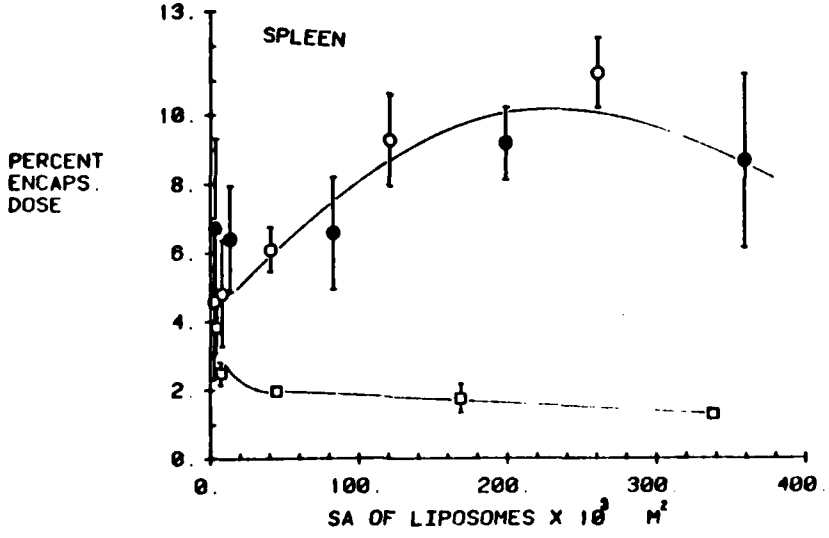
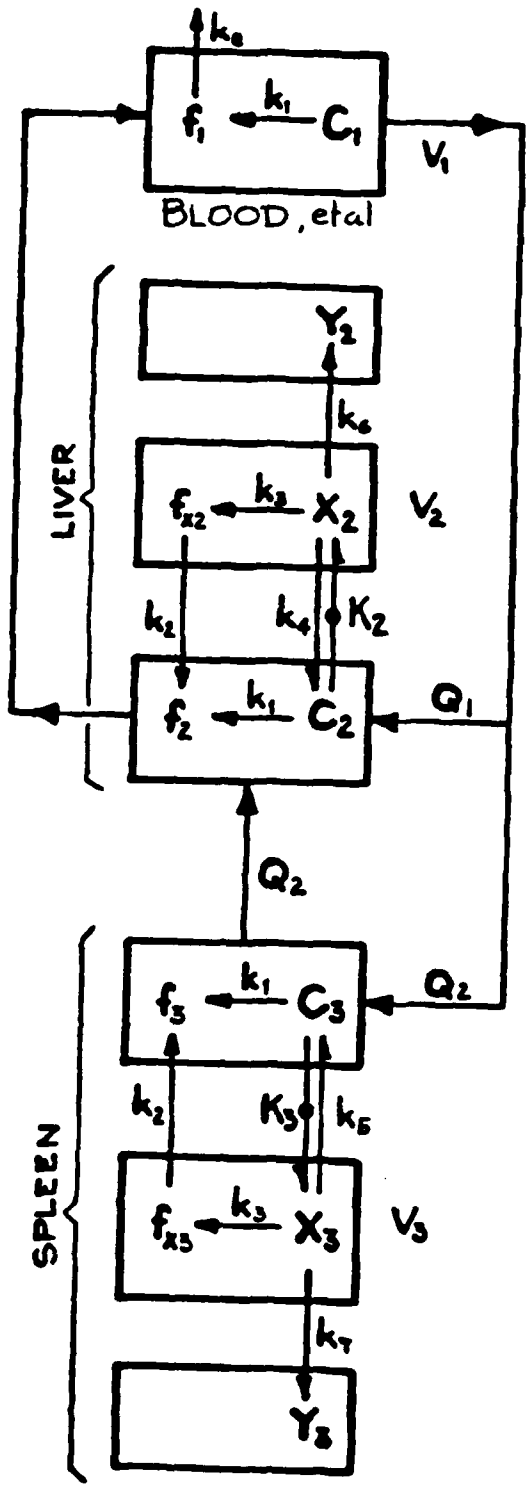


FIGURE 3. Percent of encapsulated ^{14}inulin dose in spleen. See Fig. 1 for additional details. Unlike blood and liver this data shows the most internal consistancy when plotted vs surface area of injected dose.

0 PARAMETER NAME	1 INITIAL VALUE *
1. Dose	.1
2. V_1 (ml)	4.86
3. k_e (hr^{-1})	2.7
4. k_1	.5
5. Q_1 (ml/hr)	103.
6. $V_{max,2}$	10.
7. $K_{m,2}$	.25
8. k_4	.01
9. k_3	1.
10. k_1	.5
11. k_6	.4
12. V_2 (ml)	.13
13. Q_2 (ml/hr)	5.4
14. V_3 (ml)	.01
15. $V_{max,3}$	500.
16. $K_{m,3}$	25.
17. k_5	.01
18. k_3	1.
19. k_1	.5
20. k_7	1.5

* PER 25g



LIPOSOME DISPOSITION MODEL

of free inulin. K_2 and K_3 are Michaelis constants, where $K_2=(V_{max,2}- C_2)/(K_{M,2}+ C_2)$, etc. The total blood volume, either measured or taken from the literature is $V_1 + V_2 + V_3$, where V_2 and V_3 are actual volumes of blood (literature) in liver and spleen, and V_1 is the remainder of blood. Q_1 and Q_2 are actual (literature) hepatic and spleenic blood flows. The current parameter values in use are given in the accompanying.

Discussion of why we selected this model, as opposed to some other model, would be too lengthy for this report. We know that no simplier model can account for the observed data. We know that this model is an over-simplication of reality but it can, nevertheless, account for about 85% of the variance in our data. To date our data is too limited to actually fit the model to the data, so the parameter estimates given are not unique. Further, we are limited as to what we can measure directly; for example, our measurements of liver inulin levels represent $C_2 + X_2 + Y_2 + f_2 + f_{x2}$. Yet, this model does give predicted percent dose levels quite consistent with our in vivo data. Correlations between model and observed values for liver and spleen are shown in Figures 4 and 5.

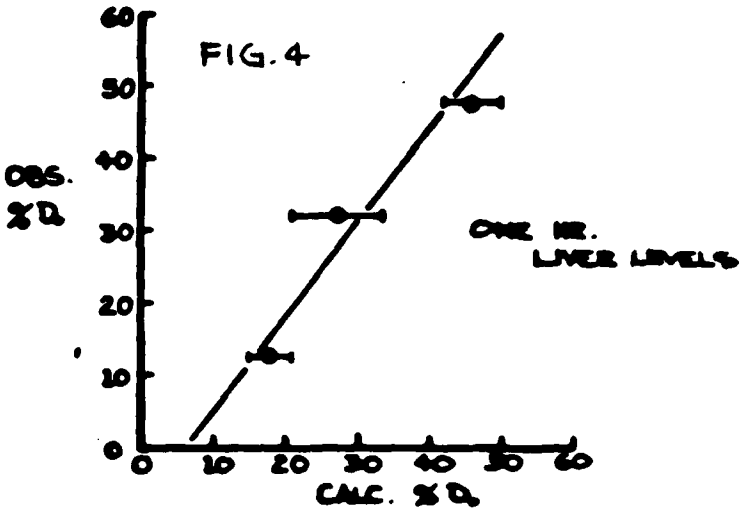
It should be noted that the value of k_1, the rate constant for leakage of liposomes in blood, was obtained directly from our in vitro plasma stability studies. Further, in order for this model to reasonably fit the in vivo data the half-life for cellular uptake by the liver is quite slow whereas for the spleen it is over three times as fast.

This model is a major step forward in understanding and predicting the in vivo properties of liposomes. But, considerable refinement remains assuming this model is reasonably accurate, we can see that as the dose of HCL approaches transfusion doses, tissue binding of all types becomes saturated, the fracting remaining in the circulation increases, and the half-life of the circulating liposomes increases.

Uptake of lipid and hemoglobin by the RES should not have any major long term toxicity (at reasonable doses, of course) since the RES is normally engaged in the uptake of both lipid and hemoglobin at all times. Yet RES uptake remains a potential problem. One aim of this proposed research is to determine at what HCL dose RES uptake, and other disposition processes, is blocked and what the consequences of such blockade may be.

We do not yet have data on transfused animals but, based on preliminary data, we can estimate the half-life of intact vesicles in the circulation at sub-transfusion doses (see Figures 6 and 7). For 0.3 HCL of the above composition at a dose of 1.4 g/Kg we can estimate that 90% of the liposomes remain in the circulation immediately after dosing and the apparent $t_{1/2}$ is 4 hrs. (Figure 6). If the dose increases by a factor of 10 the $t_{1/2}$ increases to 7.1 hrs. If the composition is changed to a less stable liposome, PC/PA/CH/aT : 4/1/2/0.1, then $t_{1/2}$ values drop to 1.6 and 2.9 hrs., respectively.

Although we have not yet maximized HCL stability in vivo, these figures demonstrate we have a good chance of designing HCL's to have the desired circulation half-lives.



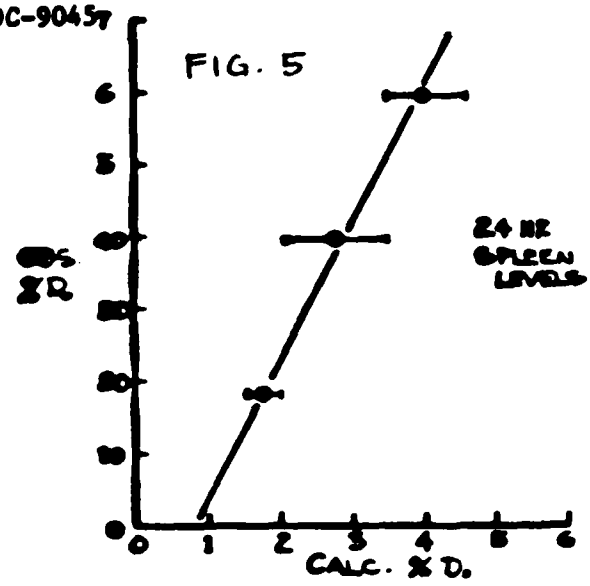
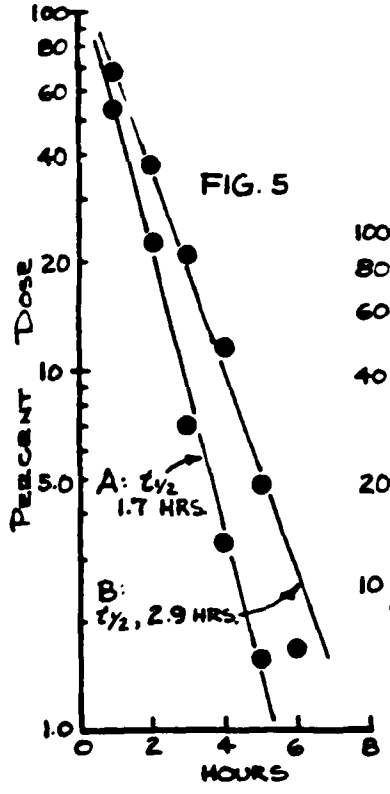


FIGURE 4. The observed one hour values for percent dose in liver for the large liposomes (Figs. 1-3) is plotted vs. the values calculated using the pharmacokinatic model. The parameters are those shown in the table. The non-zero intercept with a slope greater than unity indicates that the model is an over-simplification of reality.

FIGURE 5. The observed 24 hour values for percent dose in spleen for the large liposomes (Fig. 1-3) is plotted vs. the calculated values for the same study described in Fig. 4. See comments in Fig. 4.



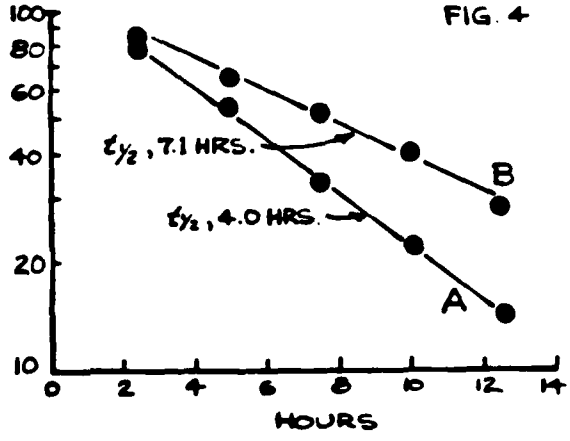


FIGURE 6. Estimation of HCL circulation half-lives. When 0.3u liposomes are composed of PC/PA/CH/aT : 4/1/5/0.1 and the dose is 1.4g (Total lipid) /Kg in mice, the apparent $t_{\frac{1}{2}}$ is 4hrs (A). Increasing the dose by a factor of 10 gives increased saturation and the apparent $t_{\frac{1}{2}}$ increases to approximately 7.1hrs (B).

FIGURE 7. Estimation of HCL circulation half-lives. When liposomes similar to those in Fig. 4 are prepared with less CH their in vivo stability decreases resulting in shorter half-times for encapsulated material (total) remaining in vivo and shorter circulation $t_{\frac{1}{2}}$ values. These estimations are for 0.3u liposomes composed of PC/PA/CH/aT : 4/1/2/0.1. For a dose of 1.4g (total lipid) / Kg the $t_{\frac{1}{2}}$ is approximately 1.6hrs (A); for a dose ten times larger the $t_{\frac{1}{2}}$ is approximately 2.9hs (B). At larger doses, such as B, circulation $t_{\frac{1}{2}}$ values are determined by the rate of in vivo liposome break-down.

CONCLUSIONS

1. We have solved the problem of scaling-up the emulsion evaporation procedure.

2. The improved procedure allows co-encapsulation of DPG with 12% SFH to yield HCL that are reasonably stable, and where P50 = 17.3 (n=2.0). We expect we can further increase both the % SFH encapsulated and the P50.

3. In vivo data on disposition of different doses of different size liposomes has been used to develop a rational, physiologically based pharmacokinetic model that reasonably accounts for HCL disposition.

4. Studies using the above system show that the circulation half-life of HCL can be controlled (by adjusting stability and size). Preliminary estimates give half-lives ranging from 1.7 to 7.1hrs.

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